

ACCELERATED PUBLICATION

Intracerebroventricular leptin regulates hepatic cholesterol metabolism

Sonya VANPATTEN*, George B. KARKANIAS†, Luciano ROSSETTI‡§ and David E. COHEN*§¹

*Department of Biochemistry, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461, U.S.A., †Department of Neuroscience, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461, U.S.A., ‡Department of Molecular Pharmacology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461, U.S.A., and §Department of Medicine, Marion Bessin Liver Research Center, Albert Einstein College of Medicine, Ullmann 625, 1300 Morris Park Avenue, Bronx, NY 10461, U.S.A.

To elucidate the control of hepatic cholesterol metabolism by leptin, rats were administered IV (intravenous) leptin, ICV (intracerebroventricular) leptin or saline. A single low dose of ICV leptin was as effective as a continuous IV infusion of high-dose leptin at decreasing the activities of 3-hydroxy-3-methyl-

glutaryl-CoA reductase and cholesterol 7 α -hydroxylase. These results indicate that the hepatic response to leptin is transduced via the central nervous system.

Key words: bile salts, cholesterol, leptin, liver, obesity.

INTRODUCTION

Reverse cholesterol transport is the metabolic pathway for movement of excess cholesterol from extrahepatic tissues to the liver for secretion into bile [1]. HDLs (high-density lipoproteins) are responsible for transport of excess cholesterol through the plasma and constitute an important source of biliary cholesterol.

Leptin is a cytokine-like hormone produced by adipocytes that reduces fat mass by decreasing food intake and increasing energy expenditure [2]. Considering that adipose tissue is a major cholesterol storage depot in the body [3], depletion of triacylglycerols by leptin necessitates transport of excess cholesterol to the liver for elimination via bile [4]. Emerging data now indicate that leptin may be an important regulator of reverse cholesterol transport. Acute IV (intravenous) administration of leptin to Zucker rats decreases hepatic biosynthesis of cholesterol and increases biliary secretion [5]. Short-term (2 days) IP (intraperitoneal) leptin administration to leptin-deficient *ob/ob* mice reverses defective hepatic HDL catabolism [6], in part by up-regulating expression of SR-BI (scavenger receptor BI) [7]. Short-term leptin administration to *ob/ob* mice also down-regulates bile salt synthesis [7], and chronic (14–28 days) IP treatment decreases both bile salt pool size and hydrophobicity [4], leading to a reduction in intestinal reabsorption of biliary cholesterol [4]. Taken together, these findings suggest that leptin orchestrates an integrated regulatory response that serves to promote the biliary elimination of adipose-derived cholesterol.

Leptin receptors are expressed in both the central nervous system and in the periphery. When introduced experimentally into the periphery by IP injection or IV infusion, leptin may interact with peripheral receptors or may be transported into the central nervous system to activate receptors in the hypothalamus [2]. To begin to dissect mechanisms in the control of reverse cholesterol transport, we compared the acute effects on hepatic cholesterol metabolism in conscious Sprague–Dawley rats undergoing IV or ICV (intracerebroventricular) leptin administration. Our results demonstrate that a single low dose of ICV leptin was as effective as a continuous IV infusion of high-dose leptin at down-regulating the hepatic activities of both HMG (3-hydroxy-3-methylglutaryl)-

CoA reductase and Cyp7A1 (cholesterol 7 α -hydroxylase). These findings strongly suggest that leptin acts within the central nervous system to promote reverse cholesterol transport.

EXPERIMENTAL

Materials

All chemicals were purchased from Sigma (St. Louis, MO, U.S.A.) unless otherwise noted. Recombinant mouse leptin was a gift from Amgen (Thousand Oaks, CA, U.S.A.). Radiochemicals were obtained from PerkinElmer (Boston, MA, U.S.A.).

Animals

Male Sprague–Dawley rats were purchased from Charles River Laboratories (Wilmington, PA, U.S.A.) and housed in the Albert Einstein College of Medicine Animal Care Facility. Rats were subjected to 12 h light/12 h dark cycles, starting at 06.00 h, and were fed a standard chow diet. Approx. 3–4 weeks prior to the day of an experiment, rats underwent surgery to implant a chronic catheter in the third cerebral ventricle (coordinates from bregma of +0.2 mm anterior/posterior, –9.0 mm dorsal/ventral, and 0.0 mm medial/lateral from the mid-sagittal suture) [8]. To prevent occlusion of the cerebral infusion catheter when not in use, a dummy cannula was inserted. During the 3-week recovery period, rats were monitored to ensure appropriate weight gain and food intake. At 3 or 4 days prior to an experiment, rats were anaesthetized and peripheral catheters were inserted into a jugular vein and a carotid artery and then exteriorized at the back of the neck. During the second recovery period, food intake was monitored and rats were weighed on the day of the experiment. These procedures were approved by the Institutional Animal Care and Use Committee of the Albert Einstein College of Medicine.

Experimental design

Experiments were carried out in fasting, conscious and unstressed rats weighing 280–330 g, with free access to drinking water. To prevent coprophagia, faeces were removed from the cages during the course of each experiment. Rats were fasted for 2 h, at which

Abbreviations used: apo, apolipoprotein; Cyp7A1, cholesterol 7 α -hydroxylase; FXR, farnesoid X receptor; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl-CoA reductase; HDL, high-density lipoprotein; ICV, intracerebroventricular; IP, intraperitoneal; IV, intravenous; LDL, low-density lipoprotein; SR-BI, scavenger receptor BI; VLDL, very-low-density lipoprotein.

¹ To whom correspondence should be addressed (e-mail dcohen@aecom.yu.edu).

point a baseline sample of blood (0.5 ml) was withdrawn from the jugular venous catheter. Rats then received one of three different treatments: (i) saline infused IV (volume matched to leptin-infused rats) and ICV (5 μ l over 5 min) (IV-saline/ICV-saline), (ii) IV leptin (bolus of 150 μ g in 1.5 ml of saline over 2 min, followed by continuous infusion at 5 μ g/min per kg) and ICV saline (5 μ l over 5 min) (IV-leptin/ICV-saline) or (iii) IV saline (volume matched to leptin-infused rats) and ICV leptin (bolus of 10 μ g in 5 μ l saline over 5 min) (IV-saline/ICV-leptin). Rats received continuous IV infusion of either saline or leptin for the entire 12 h period of the experiment using a Harvard infusion pump (Harvard Apparatus, South Natick, MA, U.S.A.). Blood (0.5 ml) was sampled at 3, 6 and 12 h following the start of an infusion. Blood samples were anticoagulated using EDTA, centrifuged at 1000 *g* for 3–4 min to remove red blood cells, and then frozen at -80°C . At the end of experiments (12 h), rats were killed by CO_2 asphyxiation. Livers were promptly removed, weighed, rinsed with PBS, snap-frozen in liquid nitrogen, and stored at -80°C for subsequent analysis.

In experiments designed to determine rates of VLDL (very-low-density lipoprotein) triacylglycerol production, blood (0.5 ml) was sampled following a 2 h fast (baseline), after which animals received ICV leptin or ICV saline as described above. Blood (0.5 ml) was sampled again at 3 h and 5.5 h and then tyloxapol (Triton WR1339) [9] was administered as an IV bolus (300 mg/kg). Thereafter blood was sampled at 15, 30, 60 and 90 min.

Analytical techniques

Plasma leptin

Plasma leptin concentrations were measured using a mouse leptin radioimmunoassay kit (Linco, St. Charles, MO, U.S.A.). According to the manufacturer's specifications, the anti-(mouse leptin) antibody quantifies rat plasma leptin concentrations with approx. 50 % efficiency compared with mouse leptin.

Plasma lipid and lipoprotein concentrations

Plasma total cholesterol and triacylglycerol concentrations were measured using enzymic assays (Sigma Diagnostics, now Sigma Aldrich). FPLC was used to separate plasma lipoproteins into VLDL, LDL (low-density lipoproteins) and HDL as previously described [5]. Cholesterol concentrations in FPLC fractions were measured enzymically (Sigma Diagnostics) in individual wells of a 96-well microtitre plate. The identities of lipoprotein fractions were determined by Western-blot analyses of apos. FPLC fractions containing purified lipoproteins were pooled and concentrated by centrifugal ultrafiltration using 10 000- M_r -cut-off filters (Centricon-10; Amicon, Beverly, MA, U.S.A.). Concentrated lipoprotein fractions were subjected to electrophoresis through SDS/4–20 % (w/v)-polyacrylamide-gradient gels (Bio-Rad, Hercules, CA, U.S.A.). Proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA, U.S.A.). Polyclonal anti-bodies (Biodesign, Saco, ME, U.S.A.) to mouse apo (apolipoprotein) A-1, apoB and apoE that were determined to cross-react with respective rat apolipoproteins, were each used at a dilution of 1:100. The secondary antibody was a goat anti-rabbit IgG (Bio-Rad). Lipoprotein cholesterol concentrations were determined as products of plasma total cholesterol concentrations and relative peak areas obtained by FPLC [5].

Tissue lipid concentrations

Concentrations of cholesterol and triacylglycerol in liver tissue were determined enzymically [4].

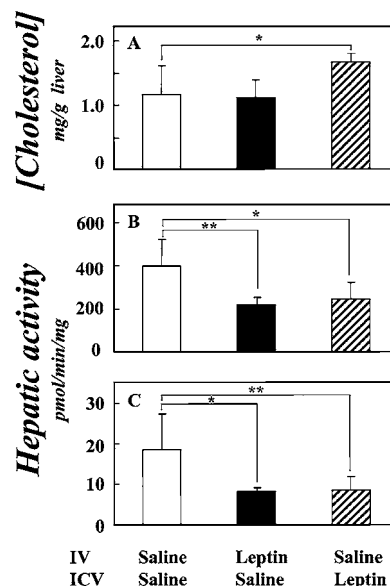


Figure 1 Regulation of hepatic cholesterol metabolism by leptin

(A) Hepatic cholesterol concentrations 12 h after administration of IV-saline/ICV-saline (white bars, $n=8$), IV-leptin/ICV-saline (black bars, $n=5$), or IV-saline/ICV-leptin (hatched bars, $n=6$) administration. Hepatic activities of (B) HMG-CoA reductase (IV-saline/ICV-saline, $n=4$; IV-leptin/ICV-saline, $n=5$; IV-saline/ICV-leptin, $n=6$) and (C) Cyp7A1 (IV-saline/ICV-saline, $n=6$; IV-leptin/ICV-saline, $n=5$; IV-saline/ICV-leptin, $n=6$). Data represent means \pm S.D.; * $P < 0.02$, ** $P < 0.01$ versus IV-saline/ICV-saline treatment.

Hepatic enzyme activities

Microsomes were prepared from 500–1000 mg of liver [4] and stored at -80°C . Microsomal protein concentrations were determined using a protein assay reagent (Bio-Rad) with BSA as a standard. Microsomes were used to measure activities of HMG-CoA reductase (EC 1.1.1.3.4) and Cyp7A1 (EC 1.14.13.17) [4].

Statistical analyses

ANOVA was employed to detect differences among mean values. Pairwise differences among means were assessed according to the Fisher's protected least-squares-differences method.

RESULTS

No differences in plasma leptin concentrations (mean \pm S.D.) were observed among the groups of rats at baseline (IV-saline/ICV-saline, 1.43 ± 0.01 ng/ml, $n=6$; IV-leptin/ICV-saline, 1.05 ± 0.27 ng/ml, $n=5$; IV-saline/ICV-leptin, 1.15 ± 0.24 ng/ml, $n=6$). For rats infused with IV-saline/ICV-saline, leptin concentrations at 12 h were unchanged compared with baseline. Plasma leptin concentrations in rats administered IV-leptin/ICV-saline were markedly elevated (545.5 ± 10.0 ng/ml). By contrast, plasma leptin concentrations in IV-saline/ICV-leptin-treated rats remained unchanged from baseline values.

The effects of peripheral and central leptin administration on hepatic cholesterol concentrations and enzyme activities are shown in Figure 1. Hepatic cholesterol concentrations in IV-leptin/ICV-saline-treated rats were not significantly different from IV-saline/ICV-saline controls (Figure 1A). By contrast, there was a 1.4-fold increase in hepatic cholesterol concentrations in IV-saline/ICV-leptin-treated animals. As demonstrated in Figure 1(B), IV and ICV leptin treatment reduced hepatic activities of HMG-CoA reductase by 1.9- and 1.6-fold respectively compared

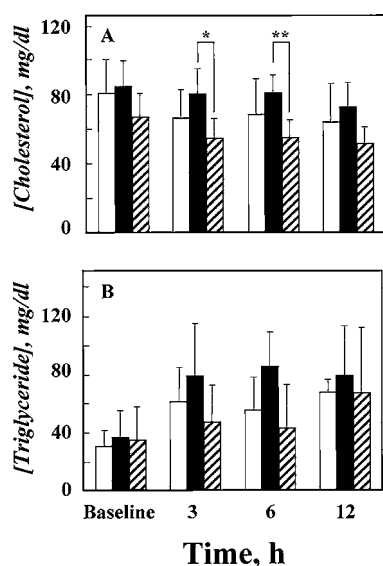


Figure 2 Influence of leptin on plasma lipid concentrations

(A) Cholesterol and (B) triacylglycerol ('Triglyceride') concentrations in plasma of rats at baseline and 12 h after administration of IV-saline/ICV-saline (white bars: cholesterol, $n=6$; triacylglycerols, $n=7$), IV-leptin/ICV-saline (black bars: cholesterol, $n=5$; triacylglycerols, $n=4$), or IV-saline/ICV-leptin (hatched bars: cholesterol, $n=6$; triacylglycerols, $n=6$). Data represent means \pm S.D.; * $P < 0.02$, ** $P < 0.01$ versus IV-saline/ICV-saline treatment.

with saline-treated rats. Activities of Cyp7A1 (Figure 1C) were reduced 2.3-fold by IV leptin and 2.2-fold by ICV leptin.

We next examined the influence of leptin on plasma lipid concentrations (Figure 2). At 3 and 6 h following leptin administration, ICV leptin decreased plasma total cholesterol concentrations compared with IV leptin infusion, which led to non-

significant ($P < 0.07$) increases in cholesterol concentrations at the same time points (Figure 2A). Neither ICV nor IV leptin administration affected plasma triacylglycerol concentrations (Figure 2B).

To determine whether the method of leptin administration influenced steady-state cholesterol concentrations of individual lipoprotein particle populations, plasma samples were fractionated by FPLC. Identification of lipoproteins in peak cholesterol-containing fractions was accomplished by Western-blot analysis. VLDL was eluted as a distinct peak in the void volume and contained apoB100 and apoB48. The next peak was determined to be LDL, as evidenced by its size and the presence of apoB100 and apoB48. A combination of LDL and HDL₁ (a large, lipid-rich subfraction of HDL) was eluted next, which included apoB100 and apoB48, apoE and trace levels of ApoA-1. The final peak was identified as HDL, because it contained apoA-1 and apoE, but no ApoB100 or ApoB48. Lipoprotein cholesterol concentrations are shown in Table 1. These values, which were obtained by integration of FPLC peak areas, were subject to considerable data scatter, as evidenced by the apparent decrease in baseline LDL cholesterol concentrations in the group of rats subsequently treated with ICV leptin. However, consistent with trends in plasma total cholesterol concentrations (Figure 2A), LDL cholesterol concentrations were higher in IV leptin- than in saline-treated rats at 3 and 6 h, and lower in rats administered ICV leptin at 6 and 12 h. HDL cholesterol concentrations were also reduced at 12 h in rats treated with ICV compared with IV leptin.

Previous experiments utilizing bile-duct-cannulated Zucker rats suggested that IV leptin may have promoted hepatic cholesterol accumulation by inhibiting VLDL production, and this in turn down-regulated HMG-CoA reductase [5]. To explore whether ICV leptin administration may have reduced VLDL production under the current experimental conditions, we measured rates of accumulation of triacylglycerols in plasma following administration of tyloxapol. Figure 3(A) shows plasma triacylglycerol concentrations of a representative animal before and after

Table 1 Influence of leptin on plasma lipoprotein cholesterol concentrations

Time (h) ...	[Cholesterol] (mg/dl)							
	Baseline	<i>n</i>	3	<i>n</i>	6	<i>n</i>	12	<i>n</i>
VLDL								
IV-saline/ICV-saline	2.0 \pm 1.1*	5	2.2 \pm 1.3	5	2.2 \pm 2.0	5	1.8 \pm 1.5	7
IV-leptin/ICV-saline	2.7 \pm 1.2	5	3.4 \pm 1.8	5	3.7 \pm 2.4	5	2.8 \pm 2.5	5
IV-saline/ICV-leptin	2.2 \pm 1.3	6	nd†	–	2.9 \pm 1.2	4	2.2 \pm 0.6	6
LDL								
IV-saline/ICV-saline	19.3 \pm 3.5	5	15.4 \pm 1.4	5	17.6 \pm 1.8	5	17.5 \pm 4.7	7
IV-leptin/ICV-saline	20.5 \pm 4.6	5	21.8 \pm 4.9‡	5	22.8 \pm 4.6‡	5	20.6 \pm 6.3	5
IV-saline/ICV-leptin	14.7 \pm 1.3‡§	6	nd	–	13.4 \pm 2.3§	4	14.1 \pm 2.5§	6
LDL/HDL₁								
IV-saline/ICV-saline	23.0 \pm 14.5	5	19.4 \pm 11.2	5	19.2 \pm 11.1	5	16.0 \pm 10.2	7
IV-leptin/ICV-saline	22.9 \pm 7.7	5	21.0 \pm 6.2	5	20.1 \pm 4.2	5	16.8 \pm 3.2	5
IV-saline/ICV-leptin	15.1 \pm 7.7	6	nd	–	13.6 \pm 6.9	4	10.0 \pm 4.7	6
HDL								
IV-saline/ICV-saline	36.0 \pm 5.8	5	29.4 \pm 4.9	5	29.6 \pm 7.8	5	28.9 \pm 8.4	7
IV-leptin/ICV-saline	38.3 \pm 8.3	5	33.9 \pm 7.1	5	34.3 \pm 5.6	5	32.3 \pm 5.8	5
IV-saline/ICV-leptin	29.5 \pm 11.7	6	nd	–	25.4 \pm 4.1	4	22.5 \pm 5.1§	6

* Values are means \pm S.D.

† nd, not determined.

‡ $P < 0.05$ versus IV-saline/ICV-saline.

§ $P < 0.05$ versus IV-leptin/ICV-saline.

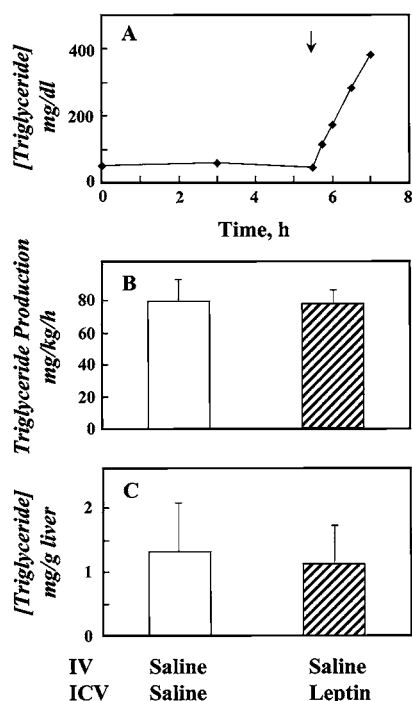


Figure 3 No influence of leptin on hepatic triacylglycerol ('Triglyceride') production or storage

(A) Plasma triacylglycerol concentrations in a representative animal before and after tyloxapol administration. The arrow indicates administration IV of 300 mg/kg tyloxapol. (B) Hepatic triacylglycerol production rates were calculated from slopes of regression lines following tyloxapol administration for rats treated with ICV-saline (white bar; $n = 4$) or ICV-leptin (hatched bar; $n = 5$). (C) Hepatic concentrations after 12 h of IV-saline/ICV-saline (white bar; $n = 8$), IV-saline/ICV-leptin (hatched bar; $n = 6$). Data represent means \pm S.D.

administration of tyloxapol. Consistent with inhibition of lipolysis by tyloxapol, plasma triacylglycerol concentrations increased linearly as a function of time. Rates of triacylglycerol production were calculated from average slope values using linear regression analyses, assuming the plasma volume to be 3.5% of total body weight [9]. As shown in Figure 3(B), triacylglycerol production was not influenced by ICV leptin administration. Whereas rates of triacylglycerol accumulation following tyloxapol administration are considered to be a more sensitive indicator of VLDL production, Ishikawa and Fidge [10] have demonstrated that most ($\approx 90\%$) of the excess cholesterol that accumulates in rat plasma is also contained in VLDL. As was the case for triacylglycerols, we observed no difference in the rate of cholesterol accumulation in plasma in response to tyloxapol (ICV-saline 68 ± 32 mg/h per kg; ICV-leptin 71 ± 26 mg/h per kg). Figure 3(C) shows that hepatic triacylglycerol concentrations did not differ in rats treated with IV-saline/ICV-saline and IV-saline/ICV-leptin. Taken together, these data indicate that ICV administration of leptin did not influence VLDL production rates.

DISCUSSION

Data from several studies indicate that leptin co-ordinates key steps in the reverse transport and biliary elimination of adipose-derived cholesterol. Administration of leptin to Zucker rats [5] and *ob/ob* mice [4,6,7] promotes hepatic uptake of HDL cholesterol, decreases cholesterol biosynthesis and reduces rates of bile salt synthesis.

Whereas hepatic cholesterol accumulation induced by leptin (Figure 1A and [4]) would be expected to down-regulate HMG-CoA reductase (Figure 1B and [5]), up-regulation of Cyp7A1 activity might have been anticipated under these conditions owing to activation of the liver X receptor [11]. Leptin instead decreased activity of Cyp7A1 (Figure 1C and [7]), indicating that a distinct mechanism was responsible. Our prior experiments in *ob/ob* mice [4] argue against activation of the FXR (farnesoid X receptor) as the mechanism by which leptin suppresses Cyp7A1 activity. This is because leptin treatment resulted in a marked decrease in the biliary content of hydrophobic bile salts, which are potent activators of FXR [11].

To gain additional mechanistic insights into the control of hepatic cholesterol homeostasis, we designed this study to determine whether the regulatory effects of leptin are transduced by receptors in peripheral tissues or in the hypothalamus. The key findings are that ICV administration of a single low dose of leptin was sufficient to reproduce the same magnitudes of down-regulation in the activities of HMG-CoA reductase and Cyp7A1 that were achieved by sustained IV administration at pharmacological doses.

Using a similar experimental design in rats [8] and mice [12], it has been demonstrated that ICV leptin sensitizes the liver to insulin action, as evidenced by inhibition of hepatic glucose production and redistribution of glucose fluxes. Because insulin down-regulates Cyp7A1 in cultured hepatocytes [13], as well as *in vivo* [14], an increase in hepatic insulin sensitivity provides a plausible mechanism by which ICV leptin decreased Cyp7A1 activity in our experiments (Figure 1C).

By contrast, the previously described effect of insulin action on the liver was to increase activity of hepatic HMG-CoA reductase [15]. Instead, down-regulation of HMG-CoA reductase following ICV leptin administration (Figure 1B) most likely occurred in response to hepatic cholesterol accumulation (Figure 1A). Consistent with the decline in plasma total cholesterol concentrations (Figure 2A), plasma was the most likely source of the excess cholesterol in the liver, possibly owing to enhanced hepatic clearance of LDL or HDL (Table 1). Considering that leptin promotes hepatic uptake of plasma cholesterol [6], due in part to up-regulation of SR-BI [7], an increased flux of cholesterol into the liver would provide a mechanistic explanation for the observed decrease in HMG-CoA reductase activity. The tendency for plasma total and LDL cholesterol concentrations to increase in rats treated with IV leptin (Figure 2A and Table 1) may reflect mobilization of cholesterol from fat in response to hydrolysis of triacylglycerols [4] that was not completely compensated for by increased hepatic clearance, such as apparently occurred with ICV leptin treatment.

Whereas these results appear to contradict our previous finding of a selective reduction in VLDL cholesterol concentrations in leptin- compared with saline-infused Zucker rats [5], it is important to consider key differences in experimental design. In that study, rats were subjected to anaesthesia and biliary diversion, whereas in the present study, rats were conscious and there was no biliary drainage. Appreciating that biliary diversion increases hepatic VLDL production [16], we speculated that down-regulation of HMG-CoA reductase occurred because leptin reduced the export of hepatic cholesterol into plasma. In the present study, steady-state VLDL cholesterol concentrations were not influenced by leptin (Table 1). However, this did not, *a priori*, exclude the possibility that changes in production were balanced by altered clearance rates from the plasma. Further investigation (Figure 3) excluded decreased production of VLDL as a source of hepatic cholesterol accumulation.

In conclusion, we have demonstrated potent effects of ICV leptin on the activities of key enzymes of cholesterol

homeostasis. In obese humans, high plasma leptin concentrations are most often associated with resistance to leptin action [2]. Current evidence in rats suggest that leptin resistance is readily acquired during overfeeding [17] and that, during weight loss, sensitivity to leptin appears to be restored well before fat mass is reduced [18]. Whereas human obesity is associated with increased rates of hepatic cholesterol and bile salt synthesis [19], these are both decreased in obese subjects during weight loss [20]. If sensitivity to endogenous leptin in humans is restored after only modest weight loss, our current observations could provide a plausible mechanism by which endogenous leptin decreases cholesterol and bile salt synthesis. These findings further suggest that efferent pathways from the hypothalamus, which control satiety and promote glucose and triacylglycerol utilization [2], may also co-ordinate cholesterol elimination by the liver.

This work was supported in part by grants DK48873 and DK56626 (to D. E. C.) from the NIH (National Institutes of Health), and grant DK20514 from the Albert Einstein College of Medicine Diabetes Research Training Center. S. V. P. was supported by an NIH training grant (GM 07491). We thank Dr Bing-Q. Liu for performing the surgical procedures and Dr Michael McCaleb (Amgen, Thousand Oaks, CA, U.S.A.) for providing the leptin used in this study.

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